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Overactivation as a Crossroads to Cancer**

PRINCIPAL INVESTIGATOR: **Juan Manuel Schvartzman, MB/BChir**

CONTRACTING ORGANIZATION: **Weill Medical College of Cornell
University, New York, NY 10021**

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14. ABSTRACT The current proposal aims to investigate the relationship between loss of classic tumor suppressor mechanisms of the Rb pathway and chromosomal instability (CIN) as controlled by the Spindle Assembly Checkpoint (SAC). Deregulation of the SAC as a result of overexpression of the Mad2 gene has already been shown to lead to the formation of a variety of solid tumors, among them breast cancer. As cells proceed from G1 to S, pocket proteins of the Rb family are inhibited and E2F factors are able to upregulate the expression of downstream targets, among them Mad2. The Rb pathway is frequently deregulated in human tumors and, in animal and cell culture models, loss of Rb function leads to high levels of Mad2. These findings lead to our hypothesis that inhibition of pocket protein function, by upregulating Mad2 levels, can drive CIN as a result of overactivation of the SAC. Here we present evidence that upregulation of Mad2 is necessary for the chromosomal instability seen after inhibition of the Rb pathway. Furthermore, we show that Mad2 normalization markedly decreases the growth of subcutaneously implanted transformed TKO fibroblasts and that, in an in vivo model of breast cancer triggered by inhibition of the Rb pathway, Mad2 heterozygosity prolongs tumor onset and leads to a shift towards a less aggressive, more differentiated tumor phenotype. These results reinforce the notion that chromosomal instability is an intricate part of tumor development and can result directly from the inhibition of a tumor suppressor pathway. We are currently investigating whether later events in the tumorigenic process are also affected by chromosomal instability.					
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Abstract

The current proposal aims to investigate the relationship between inhibition of the Rb tumor suppressor pathway and chromosomal instability (CIN) as controlled by the Mitotic Checkpoint (MC). Deregulation of the MC as a result of overexpression of the Mad2 gene has already been shown to lead to the formation of a variety of solid tumors, among them breast cancer. As cells proceed from G1 to S, pocket proteins of the Rb family are inhibited and E2F factors upregulate the expression of downstream targets, among them Mad2. The Rb pathway is frequently deregulated in human tumors and, in animal and cell culture models, loss of Rb function leads to high levels of Mad2. These findings suggest that inhibition of pocket protein function, by upregulating Mad2 levels, can drive CIN by way of MC overactivation. Here we present evidence that upregulation of Mad2 is necessary for the chromosomal instability seen after inhibition of the Rb pathway. We show that Mad2 normalization markedly decreases the growth of subcutaneously implanted Rb family null transformed fibroblasts and that, in a transgenic mouse model of breast cancer driven by inhibition of the Rb pathway, Mad2 heterozygosity prolongs tumor onset and leads to a shift towards a less aggressive, more differentiated tumor phenotype. These results reinforce the notion that chromosomal instability is an intricate part of tumor development and can result directly from the inhibition of a tumor suppressor pathway. We are currently investigating whether later events in the tumorigenic process are also affected by chromosomal instability.

Introduction

The impaired segregation of sister chromatids to daughter cells during cell division, chromosomal instability (CIN), is a phenomenon intricately linked to tumor development. Until recently, whether CIN played a causative role in tumor initiation and progression or whether it was simply a passenger phenotype were largely unknown. Recent work from our laboratory and others has since shown that in fact CIN on its own can initiate tumor formation in a large variety of tissues, among them breast [1-3]. Nevertheless, while mutations that lead to impaired DNA damage checkpoints have been found in a variety of tumors, the molecular components of the mitotic checkpoint (Mad2, Securin, CycB1, BubR1, Cdc20, among others) are rarely mutated in clinical specimens [4]. In fact, they are often transcriptionally upregulated, an event that cell line data shows leads to mitotic checkpoint overactivation [5, 6]. Given that many of the mitotic checkpoint genes (Mad2, Securin, CENP-E, BubR1, CycB1) are targets of the transcription factor family E2F [7], and knowing that more than 50% of breast tumors have abnormalities in the Rb tumor suppressor pathway that lead to uncontrolled E2F target activation [8-16], we postulated that chromosomal instability could be an important tumor driving force that is initiated solely as a result of Rb pathway inhibition. Here we present preliminary data showing that Mad2 upregulation as a result of Rb pathway inhibition is sufficient to generate chromosomal instability and plays a significant part in the development of mammary adenocarcinomas in a transgenic murine model.

Body

Specific Aim 1: Pocket protein loss and chromosomal instability as a result of Mad2 upregulation

In order to address whether Mad2 upregulation upon inhibition of pocket protein activity can mediate chromosomal instability, we took advantage of Murine Embryonic Fibroblasts (MEFs) derived from Embryonic Stem Cells where all three pocket protein genes (Rb, p107 and p130) have been deleted by homologous recombination (a gift from Dr. Julian Sage [17]). Preliminary analysis showed that these cells expressed significantly higher levels of Mad2 as compared to wild type and single or double knock-out MEFs of similar passages (Figure 1). This confirms that Mad2 is an E2F target whose expression is under control in some measure by all three pocket proteins. In order to determine whether the observed Mad2 upregulation was necessary for CIN and tumorigenic effects, we sought to normalize the levels of Mad2 to those approaching wild type. Using a panel of lentiviral vectors expressing short-hairpin RNAs, we found two constructs that reduced the levels of Mad2 to the desired wild type range (Figure 1). Normalization of Mad2 levels in TKO cells resulted in a modest decrease in proliferation rate as determined by growth curves and 3T3 assays (Figure 2). Importantly, this decreased proliferation was not the result of apoptosis nor senescence, as determined by AnnexinV staining and Senescence Associated β -Gal (data not shown). Furthermore, almost complete knockdown of Mad2 was cell lethal, with no cells surviving past 5 passages after puromycin selection for transduced cells (Figure 2). This is in agreement with other reports that have failed to show any viability after complete downregulation of Mad2.

As outlined in the introduction, if Mad2 upregulation was responsible for CIN in TKO cells, its normalization would be expected to result in a rescue of this phenotype. Support for this hypothesis was the fact that karyotypes of passage 25 TKO cells with normalized levels of Mad2 were significantly less variable than those of control TKO cells (Figure 3). Of twenty cells analyzed in each condition, the spread of chromosome counts in control TKO cells was substantially broader than that of shMad2 TKO cells and, furthermore, none of the TKO cells had the same chromosome complement while more than half of them did in the normalized Mad2 TKO cells. Nevertheless, this decrease in variability could be a result of outgrowth of a particularly resistant clone of cells or a decreased proliferative rate. To control for these events, we developed an assay of chromosomal instability using Fluorescent in situ Hybridization with chromosome specific probes (chFISH) of single cell colonies that have been grown to 150-500 cells. A frequency distribution analysis of each colony for cells of each complement (0 to 6 or more signals for a chromosome 12 probe) revealed a significant decrease in the deviation from the mode in Mad2 normalized cells compared to TKO controls (Figure 3). This strongly suggests that Mad2 upregulation is required for the chromosomal instability observed in cells with an absent Rb pathway.

TKO cells that have been transduced with an HrasV12 retroviral vector form fibrosarcomas when intradermally implanted into the flanks of nude mice. In order to determine whether chromosomal instability as a result of Mad2 overexpression played a part in the growth of these tumors, we compared the growth rates of TKO HrasV12 intradermally implanted cells with those

of TKO shMad2 HrasV12 cells. As can be observed, we found a significant decrease in the growth kinetics of these tumors when Mad2 levels were normalized (Figure 2). The effects may even be an underestimate of the real effect as in our hands lentiviral vectors are progressively silenced when transduced cells are grown in the absence of the selection agent puromycin, which we did not administer to nude mice.

In our initial Statement of Work we proposed to generate chimeric mice derived from wild type and TKO ES cells, and to study the effects of Mad2 heterozygosity on the tumor phenotype observed in these chimeras. Due to the difficulty in obtaining the different strains required and the time required for the serial breedings, we opted to normalized Mad2 levels in TKO ES cells by lentiviral transduction with the vectors used in the previous section. Unfortunately, transduction of TKO ES cells with shMad2 lentiviral vectors did not lead to significantly reduced Mad2 levels, possibly as a result of lentiviral silencing. In light of the progress of our *in vivo* transgenic model, we have opted to discontinue section 1.2.

WAP-T121 transgenic animals develop mammary adenocarcinomas with a mean latency of 350 days as a result of inhibition of pocket proteins by the T121 fragment of large T-antigen [18]. We have confirmed that mammary glands of T121 animals have substantially higher levels of Mad2 protein and that WAP-T121;Mad2^{+/-} animals have Mad2 levels similar to wild type (Figure 4). If Mad2 upregulation is required for the tumor phenotype observed in WAP-T121 animals, we would expect a decrease in tumor incidence or progression in the Mad2^{+/-} background. In fact, the median latency was prolonged to 409 days in this setting, a result that was statistically significant. Furthermore, the tumor spectrum observed was significantly different, with Mad2 wild type animals showing more aggressive tumors and a larger tumor burden per animal. We are currently carrying out chromosome FISH analysis of tumor sections to determine if, as expected, Mad2^{+/-} animals show decreased aneuploidy in tumors. In addition, tumor cells can be grown for a limited number of passages *ex vivo*, allowing us to transduce WAP-T121 tumor cells with lentiviral short hairpin vectors in order to decrease Mad2 levels and determine if growth of orthotopically transplanted cells is impaired or if later tumorigenic events, such as metastasis, are affected.

Specific Aim 2: Securin as a mediator of Mad2 induced tumorigenesis

In order to determine if Securin overexpression is sufficient to initiate tumorigenesis in a transgenic animal model, we have generated TRE-Securin mice that overexpress wild type Securin cDNA only when a doxycycline dependent transactivator is present and doxycycline is provided in the feed. As proof of principle of inducible overexpression, we have studied the behavior of MEFs in culture as well as lymphocytes isolated from double transgenic (TRE-Securin;CMV-rtTA) animals of 2 different founders. We chose to initially characterize overexpression of securing with the CMV-rtTA driver allele as this is expressed in the majority of tissues, including mammary. In culture, overexpression of Securin was evident, in some cases at very high levels, and lead to a moderate increase in aneuploidy as characterized by chromosome counts of metaphase spreads in fibroblasts and lymphocytes (Figure 5). The aneuploidy was, however, significantly lower than what we have previously observed for Mad2 overexpression, arguing that Securin is not solely responsible for the phenotypic consequences of checkpoint overactivation by Mad2.

In order to determine the consequence of Securin overexpression *in vivo*, we have generated a cohort of TRE-Securin;CMV-rtTA and TRE-Securin;CMV-tTA in which the transgene is only expressed in the presence or absence, respectively, of doxycycline. This cohort is currently under observation for any behavioural signs of clinical disease. Importantly, RT-PCR analysis confirms expression of the Securin transgene in lung and liver (Figure 5).

Mad2 overexpression results in Securin stabilization both *in vivo* and *in vitro* but whether this stabilization is required for the phenotypic consequences of Mad2 overexpression remains unknown. In order to address this, we have bred Mad2 overexpressing animals with mice that lack the Securin gene (Securin null animals were a gift from K. Nasmyth). If Mad2 overexpression requires Securin for aneuploidy and/or tumorigenesis, Securin loss in this context should rescue these phenotypes. Importantly, Securin mice are viable and are born at the expected Mendelian ratios. Cohorts of Mad2 overexpressing animals in Securin wild type and null backgrounds are being followed for the appearance of clinical disease.

Key Research Accomplishments

- Mad2 is markedly upregulated in cells lacking all three Rb family members (TKO cells). Figure 1.
- Mad2 upregulation is required for the chromosomal instability seen upon Rb pathway inhibition: normalization of Mad2 levels in TKO fibroblasts rescues the chromosomal instability observed in counterpart controls. Figure 3.
- Growth of intradermally implanted TKO cells is significantly decreased after normalization of Mad2 levels. Figure 2.
- In the WAP-T121 mammary adenocarcinoma model, Mad2 heterozygosity significantly delays tumor onset, decreases tumor burden and leads toward a shift to a more differentiated tumor spectrum. Figure 4.
- Securin overexpression in vivo leads to moderate aneuploidy in MEFs and lymphocytes. Figure 5.

Reportable Outcomes

N/A

Conclusion

Using primary cells lines lacking all three Rb family members, we have shown that Mad2 upregulation as a result of E2F overactivity is required for the chromosomal instability shown in this system. In addition, we have found that normalizing Mad2 levels leads to a marked decreased in tumor forming ability of transformed TKO fibroblasts. Similarly, in an *in vivo* model of mammary adenocarcinoma driven by inhibition of the Rb pathway, we found that Mad2 heterozygosity results in an increase in tumor free survival and a tumor spectrum shift towards a more differentiated phenotype. We are currently investigating whether differences exist between these two subgroups and, specifically, whether Mad2 upregulation impacts on later tumor events such as a metastasis. Furthermore, we are continuing our work in order to identify the putative mechanisms by which Mad2 overexpression leads to chromosomal instability and the roles of Securin stabilization in this process.

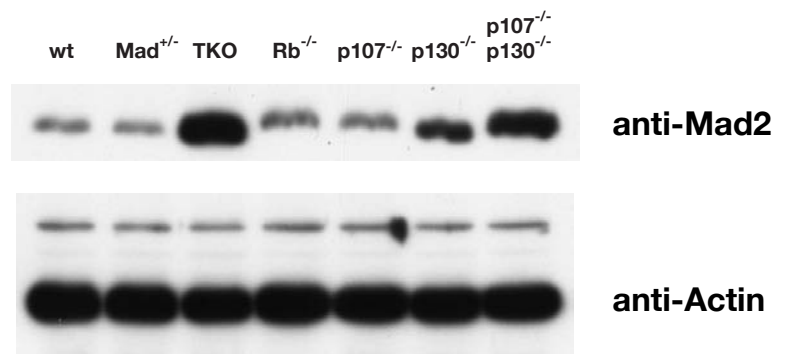
These results strongly support a tumor initiation and progression model whereby the early inactivation of key tumor suppressor pathways, by leading to inappropriate expression of mitotic checkpoint genes (in this case Mad2), can in itself lead to chromosomal instability, an event known to accelerate tumor progression. In addition, as Rb targets such as Mad2 are frequently upregulated in human tumors and are known to overactivate the mitotic checkpoint, our data argue that checkpoint overactivation, rather than loss or mutation of checkpoint components, as proposed by a number of other groups, is the key contributor to genomic instability in solid aneuploid tumors.

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Figure 1



lentiviral shMad2 vectors in wt cells

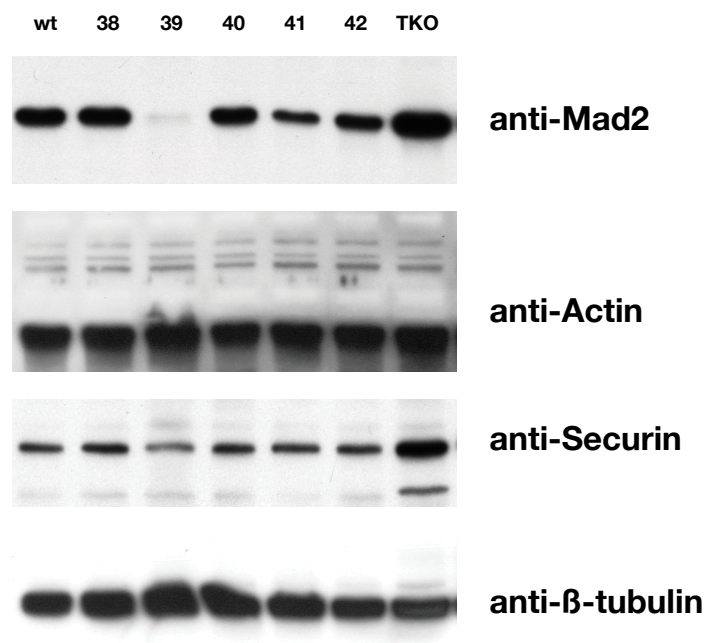


Figure 1. Top: Western Blot of asynchronously growing fibroblasts of indicated genotypes showing highly elevated Mad2 levels in TKO cells. Bottom: Western Blot of fibroblast lysates after transduction with lentiviral RNAi short hairpin vectors targeting Mad2. Securin levels confirm decreased mitotic checkpoint overactivation after Mad2 knockdown.

Figure 2

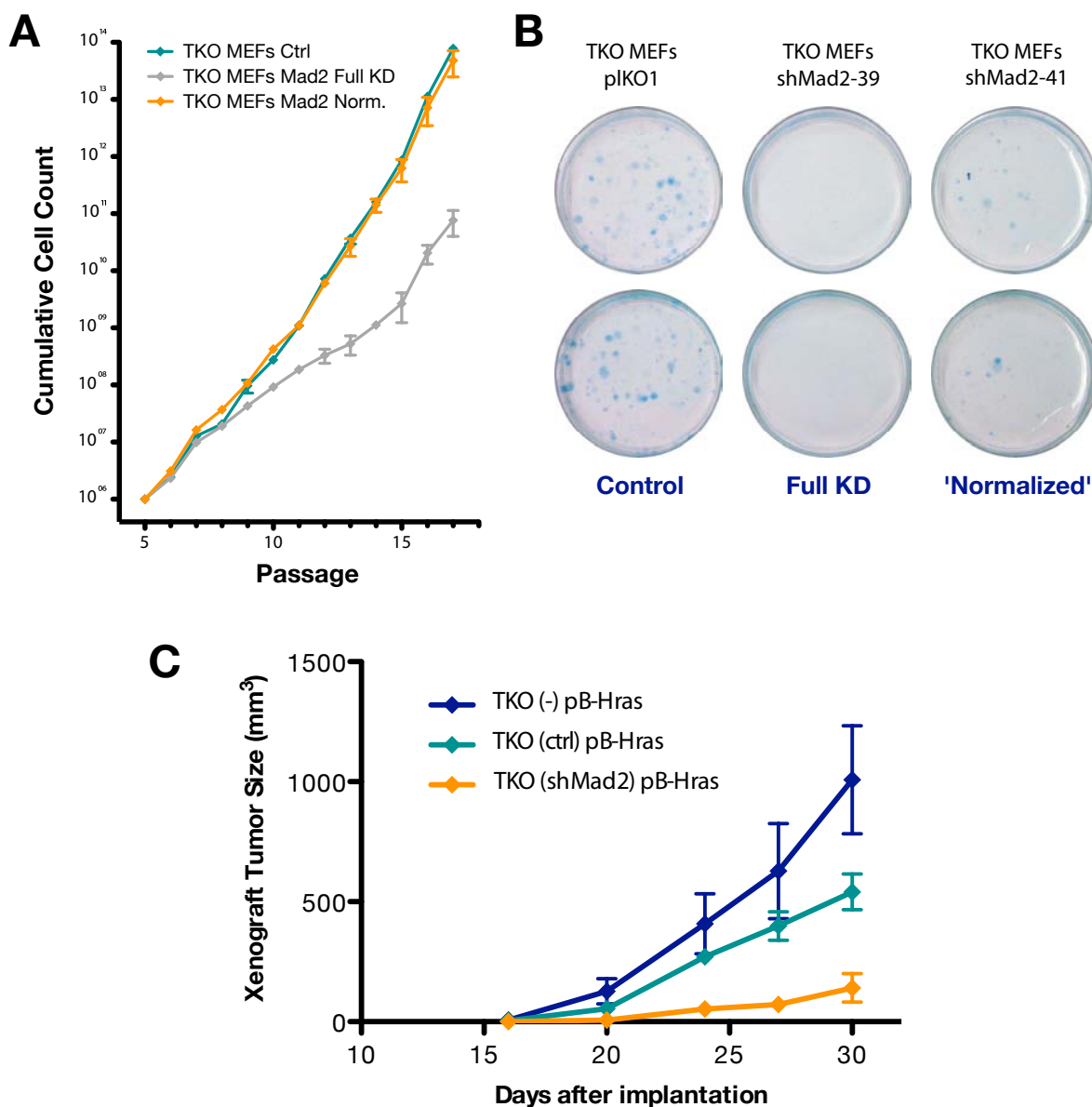


Figure 2. A. 3T3 assay showing proliferation of control, Mad2 normalized and Mad2 full knockdown TKO MEFs. Note that the full knockdown curve at later passages shows cells that have become resistant to the hairpin. B. Low seeding efficiency assay of control, Mad2 normalized and Mad2 full knockdown cells. C. Growth of intradermally implanted transformed TKO MEFs after downregulation of Mad2 levels. Tumor growth in nude mice was assayed weekly.

Figure 3

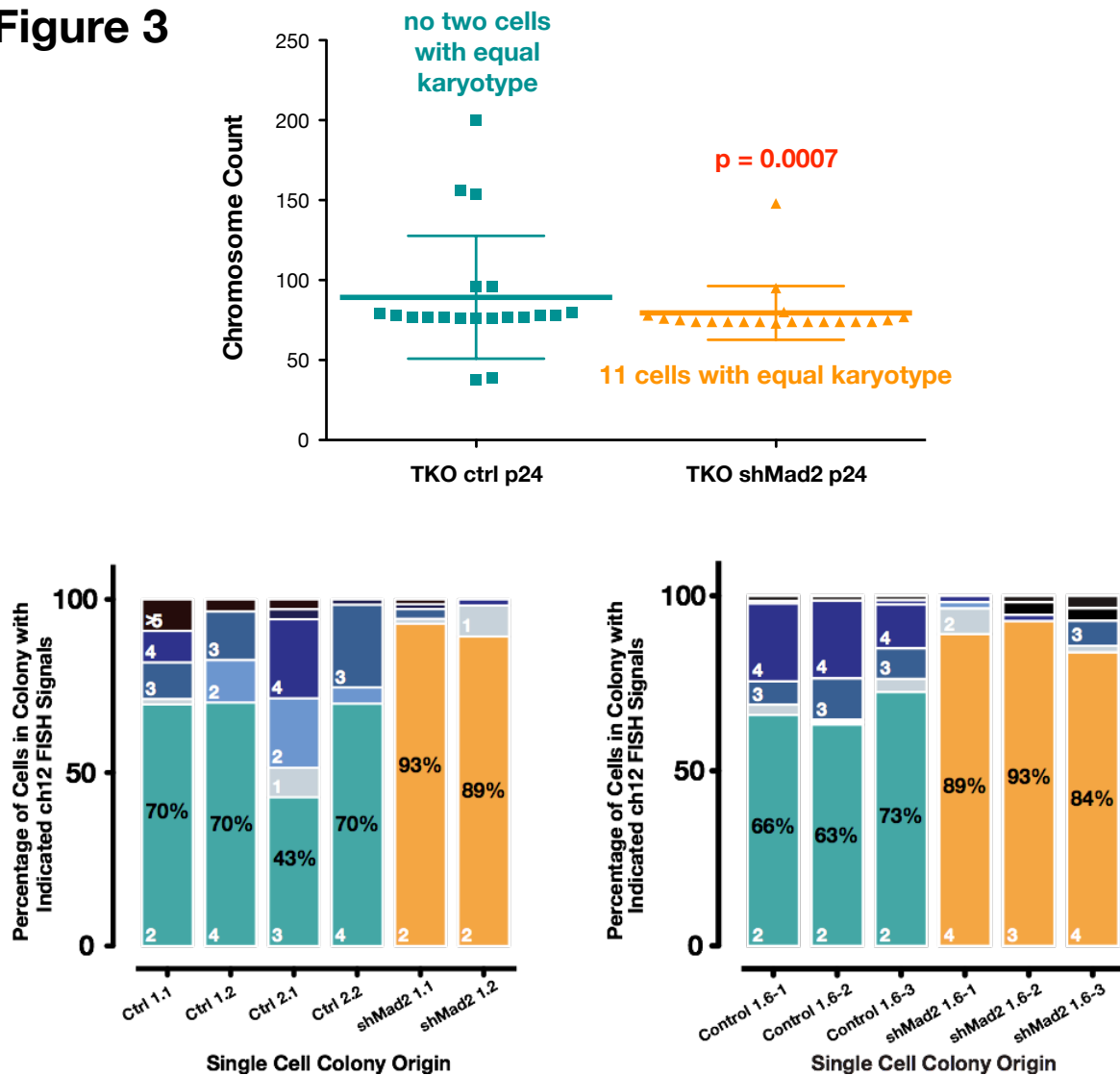


Figure 3. Top: karyotypes of late passage TKO control and Mad2 normalized MEFs showing decreased modal deviation after Mad2 normalization. Bottom: Modal and deviation plots of single cell colony chromosome FISH assays. Single cell colonies were grown to approximately 500 cells, fixed and processed with a chromosome 12 specific probe. Each bar represents one colony (control in blue and Mad2 normalized in orange). Percentages correspond to the number of cells with the respective number of signals in each colony. Differences between control and Mad2 normalized groups were significant in pairwise comparisons ($p < 0.03$) using an unpaired t-test.

Figure 4

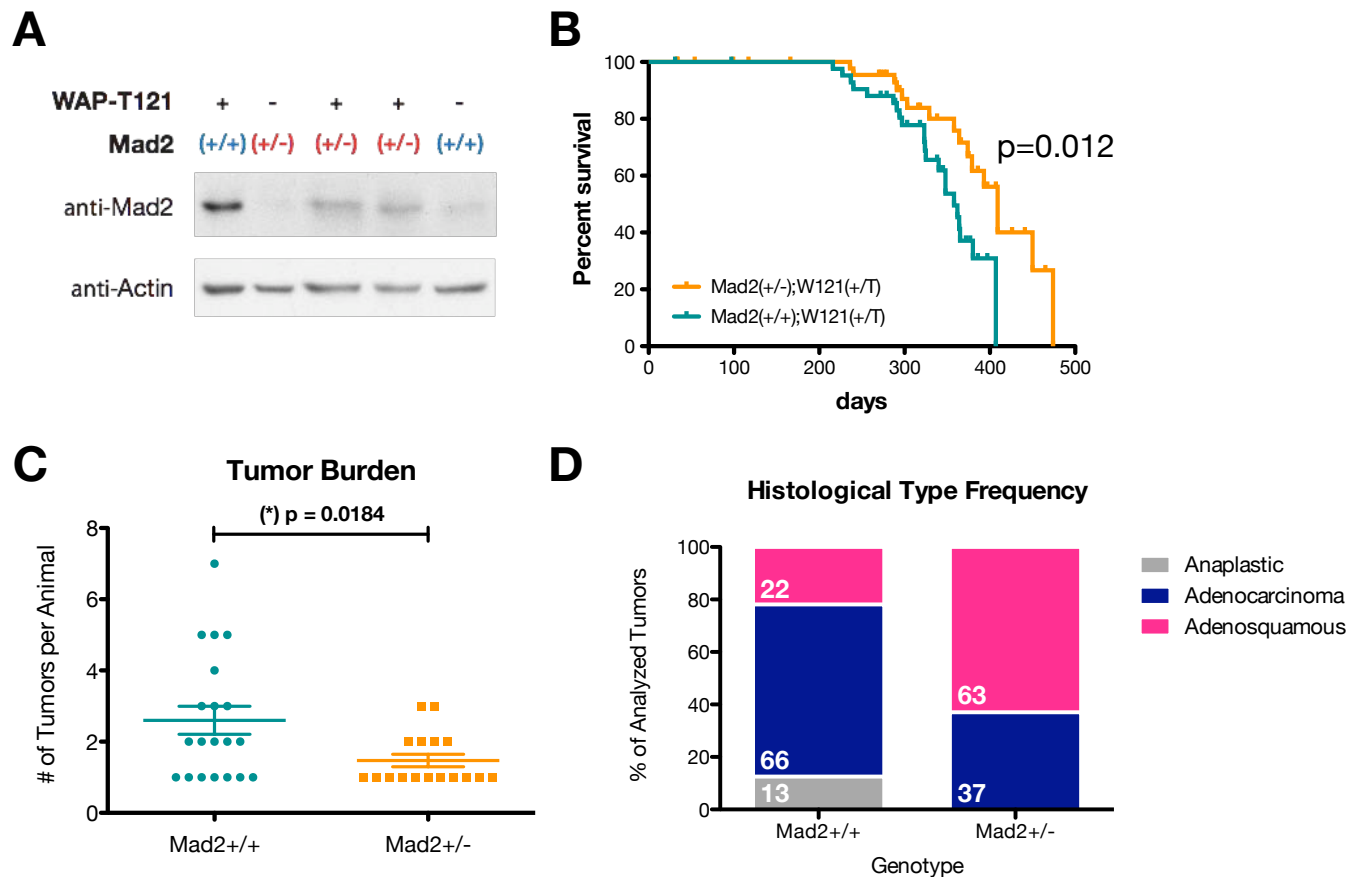


Figure 4. A. Expression levels of Mad2 protein in 10 week old female mammary glands of indicated genotypes. B. Tumor free survival curve of WAPT121 Mad2 wildtype versus heterozygote animals. C. Distribution of tumor burden per animal. D. Distribution of histological type per genotype. Adenosquamous were the most differentiated of tumors encountered in both subgroups, adenocarcinomas were intermediate and anaplastic tumors were the most aggressive.

Figure 5

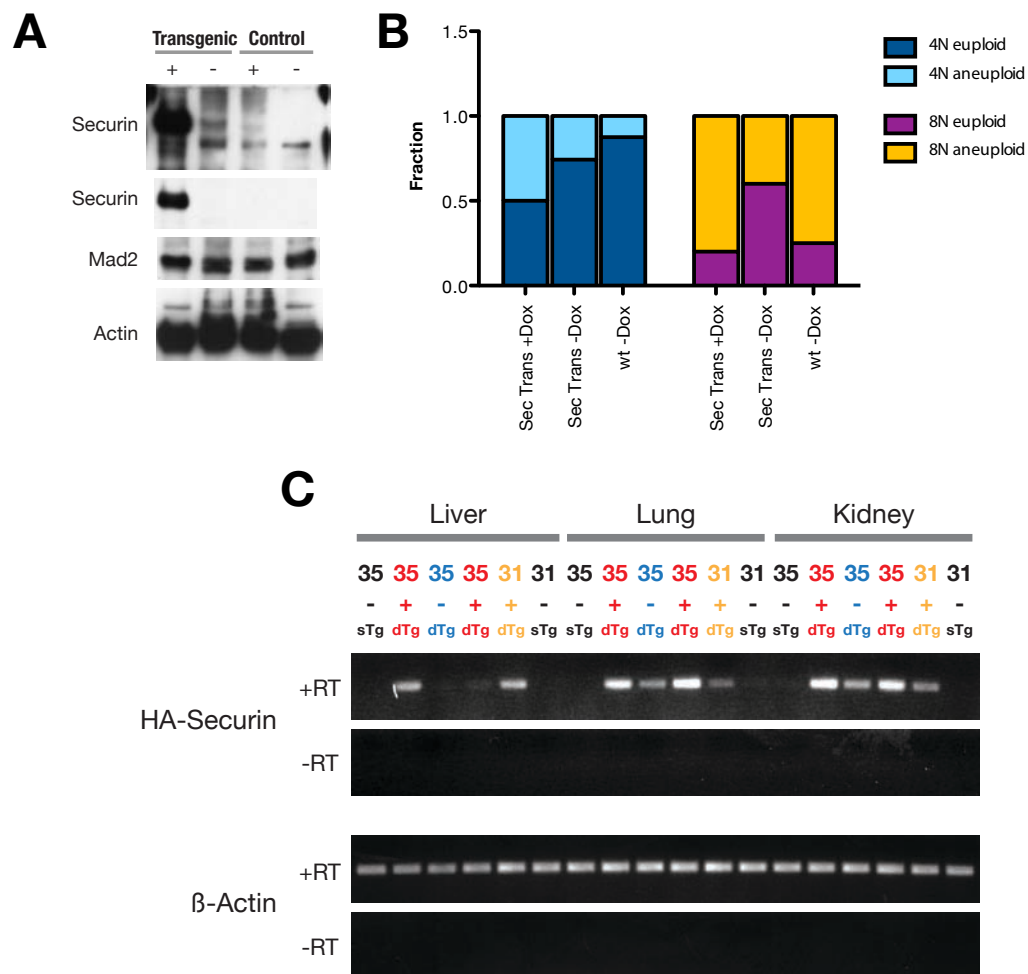


Figure 5. A. Western Blots of MEF lysates derived from transgenic TRE-HASecurin, CMV-rtTA and control CMV-rtTA animals showing inducible Securin overexpression when treated with doxycycline (+). Note the larger overexpressed band corresponding to HA-Securin. B. Aneuploidy distributions of Securin overexpressing MEFs compared to controls. C. RT-PCR of liver, lung and kidney showing inducible overexpression of Securin in two founder lines (35 and 31).